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Putrescine induces somatic embryo development and proteomic changes in embryogenic callus of sugarcane

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ABSTRACT

Somatic embryogenesis, an important biotechnological technique, has great potential for application in sugarcane breeding and micropropagation. Polyamines have been associated with the regulation of several physiological processes, including the acquisition of embryogenic competence and somatic embryogenesis. In this study, we used a proteomic approach to evaluate the effects of exogenous polyamine on sugarcane somatic embryo development to better understand this process. Embryogenic cultures were treated with different concentrations of putrescine, spermidine, and spermine. Proteomic analyses combined the shotgun method and the nanoESI-HDMS^E technology. Among polyamines, 500 μM putrescine gave rise to the highest number of somatic embryos; however, no differences in the amount of fresh matter were observed between polyamines and control. Differences in protein abundance profiles resulting from the effect of 500 μM putrescine on sugarcane somatic embryo maturation were observed. Proteomic analyses of putrescine and control treatment showed differences in the abundances of proteins related to somatic embryogenesis, such as arabinogalactan proteins, peroxidases, heat shock proteins, glutathione s-transferases, late embryogenesis abundant proteins, and 14-3-3 proteins. These results show that putrescine and the identified proteins play important roles in protecting the cells against an in vitro stress environment, contributing to the formation of somatic embryos during the maturation treatment. *Biological significance:* Despite all studies with somatic embryogenesis, the molecular mechanisms controlling the process have not been completely understood. In this study, we highlighted the effects of the polyamine putrescine on somatic embryogenesis of sugarcane and the differentially abundant proteins related to somatic embryo development. We identified six groups of important stress related proteins that are involved in the adaptation of cells to the stress environment of in vitro culture and may also be part of the mechanisms associated to the somatic embryogenesis process. Therefore, our research is trying to understand the complexity of how one single somatic cell becomes a whole plant.

1. Introduction

Currently, all of the cultivated sugarcane worldwide is derived from the crossing of two main species: *Saccharum officinarum*, a domesticated species that accumulates high sugar content and presents a basic number of chromosomes equal to 10 and a chromosome constitution of 2n = 80, and *Saccharum spontaneum*, a wild species presenting a basic number of chromosomes equal to 8 and a chromosome constitution of 2n = 40–128, which has high resistance to biotic and abiotic stresses [1–3]. The hybrid derived from this cross shows a genomic constitution even more complex than the parental genomes, featuring a chromosome constitution of 2n = 100–130, where 60–70% of the chromosomes have been inherited from *S. officinarum* [3]. Sugarcane cultures allow several means of economic exploitation, such as sugar, ethanol, and biopolymers, as well as electricity generation and cellulosic ethanol from the bagasse and straw. This species has been cultivated on an industrial scale for sugar production in more than 90 countries worldwide for over 100 years, and the interest in its cultivation has increased due to the production of ethanol as a renewable energy source [4].

The potential for the application of biotechnological tools to improve sugar production and agronomic performance of sugarcane crops is relatively promising because the yield gains using conventional breeding may be reaching their limit due to the difficulties imposed by the complex genome of sugarcane [3]. Furthermore, the selection of superior genotypes within a population obtained by crossing two individuals is a long-term project that takes at least ten years to generate results [5].

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Therefore, biotechnological tools have been considered particularly important for sugarcane crops due to the insertion of new genes conferring advantageous agronomic characteristics [6]. One possible morphogenetic pathway for this process is through somatic embryogenesis, an in vitro culture system in which a single somatic cell, or small group of cells, gives rise to a somatic embryo [7]. Several research programs using in vitro cultures of sugarcane have been conducted using various applications, such as micropropagation, breeding, germplasm conservation, and genetic engineering [3,8]. Moreover, the comparison of proteomics [9] and metabolomics profiles [10,11], dehydrin proteins [12], antioxidant enzyme activity [13], polyamine contents and morphological studies [14] between embryogenic and non-embryogenic callus in several cultivars of sugarcane has been undertaken to understand the complete route that triggers the de-differentiation, re-differentiation, and development of somatic cells into embryos.

In the process of somatic embryogenesis, various molecules, including polyamines, have been described as important induction signals in plants. The effects of polyamines on somatic embryogenesis have been described for several species [15–17], including sugarcane [14,18,19]. Polyamines have been considered a class of plant growth regulators; they are small, polycationic aliphatic molecules bearing amino groups that are capable of electrostatically interacting with macromolecules such as nucleic acids, phospholipids, cell wall components, and proteins [20,21]. Putrescine, spermidine and spermine are the main polyamines in plants, and they have been associated with the regulation of physiological processes, such as organogenesis, embryogenesis, flower development, senescence, fruit maturation and development, as well as responses to biotic and abiotic stresses [22]. In embryogenic and non-embryogenic callus from sugarcane var. SP79-1011, the changes in endogenous polyamines profile, especially in spermine contents, may be important for the acquisition of embryogenic competence and somatic embryo maturation in embryogenic callus [14].

To gain a better understanding of the biochemical, physiological, and morphological changes that these molecules may cause in plant development, proteomic tools might be useful for studying gene expression products through the identification of differentially abundant proteins and, potentially, their interactions.

The development of new technologies in the field of mass spectrometry has allowed the acquisition of reliable and high quality data, which is of particular importance for the analysis of highly complex protein mixtures. During MS^E acquisition, the mass detector alternates between a low-energy scanning mode (MS), for accurate mass peptide precursor identification, and an elevated-energy mode (MS^E), for generation of accurate mass multiplex peptide fragmentation data, from which both quantitative and qualitative characterization of complex proteomic samples can be obtained [23,24]. The use of traveling wave-based ion mobility separation (IMS) has provided an additional dimension of separation, improving system peak capacity while reducing chimeric and composite interferences, thus increasing the resolving power of the IMS-enhanced MS^E analyses (high definition MS^E, $HDMS^E$) [25].

The main objective of this work was to study the effects of exogenous polyamines on somatic embryo induction and differential abundance of proteins during the somatic embryogenesis of sugarcane cv. SP80-3280 to acquire a more comprehensive understanding of the mechanisms underlying this complex process. In our study, we used a high-throughput proteomic approach combining the shotgun method and the nanoESI-$HDMS^E$ (data-independent acquisition, with ion mobility) technology.

2. Materials and methods

2.1. Plant material

Sugarcane plants cv. SP80-3280 were obtained from the Universidade Federal Rural do Rio de Janeiro (UFRRJ), Campus Leonel Brizola, localized in Campos dos Goytacazes, RJ, Brazil (21° 48'S and 41° 17'W). This variety was chosen based on a search using The Sugarcane EST Project (SUCEST) protein databank (http://sucest-fun.org/), which helped with the acquisition of more reliable $HDMS^E$ data.

Callus induction was performed as previously described [14]. Internodes with axillary buds were planted in plastic trays containing the commercial substrate PlantMax (DDL Agroindustria, Paulínia, São Paulo, Brazil) for a period of two months. Subsequently, plants were processed by removing the mature leaves. The resulting leaf rolls were surface sterilized in 70% ethanol for 1 min, then in 30% commercial bleach (2–2.5% sodium hypochlorite) for 15 min, and subsequently washed three times in autoclaved distilled water. As explants, leaf rolls were transversely sectioned into 2–4 mm-thick slices and cultured in test tubes (150 × 25 mm) containing 10 mL of MS [26] (Phytotechnology Lab, Overland Park, KS, USA) culture medium, supplemented with 20 g/L sucrose, 2 g/L Phytagel® (Sigma-Aldrich, St. Louis, MO, USA) and 10 μM 2,4-dichlorophenoxyacetic acid (2,4-D - Sigma-Aldrich). The pH of the culture medium was adjusted to 5.8 before Phytagel was added. The culture medium was sterilized by autoclaving at 121 °C for 15 min, and after inoculation, the cultures were kept in the dark at 25 ± 1 °C.

After 45 days in culture, samples of induced callus were transferred to Petri dishes (90 × 15 mm) containing 20 mL of the same culture medium, kept in the dark at 25 ± 1 °C, and then subcultured every 21 days. During this multiplication period, embryogenic callus was separated according to morphological characteristics as previously described [14].

2.2. Effects of exogenous polyamines in somatic embryo induction

For analysis of polyamine effects on somatic embryo induction, five Petri dishes containing 20 mL of MS medium supplemented with 30 g/L sucrose and 2 g/L Phytagel were inoculated with three colonies of 200 mg fresh matter (FM) of embryogenic callus per Petri dish. The pH of the culture medium was adjusted to 5.8 before Phytagel was added. Various concentrations (0, 10, 100 and 500 μM) of the polyamines putrescine, spermidine, and spermine were used separately. Polyamine solutions were adjusted to pH 5.8 and filter-sterilized before addition into the autoclaved MS medium. The culture medium was sterilized by autoclaving at 121 °C for 15 min, and the cultures were kept at 25 ± 1 °C in the dark for 7 days and transferred to light for an additional 21 days of culture, with a photoperiod of 16 h (90 μmol/m^2/s). Before (time 0) and after 7, 14, 21, and 28 days of culture, the FM increment and the number of somatic embryos formed were evaluated from the embryogenic callus.

The best treatment in terms of the production of somatic embryos and the control treatment were utilized for subsequently polyamine and proteomic analyses. For these analyses, callus colonies were homogenized, and samples with 300 mg FM were stored at −20 °C. For polyamine analysis, samples were collected before (time 0) and after 7, 14, 21 and 28 days of culture, whereas for proteomics, samples from 14 and 28 days in culture were utilized.

The somatic embryos were regenerated on MS culture medium supplemented with 30 g/L sucrose and 2 g/L Phytagel. The pH of the culture medium was adjusted to 5.8 before Phytagel was added. The culture medium was sterilized by autoclaving at 121 °C for 15 min, and after inoculation, were incubated at 25 ± 1 °C, with a photoperiod of 16 h (90 μmol/m^2/s) for 30 days. For acclimatization, sugarcane plants were transferred to 50 mL plastic cups containing plant substrate and vermiculite (1:1) and kept at 25 ± 1 °C under a photoperiod of 16 h (90 μmol/m^2/s). Cups were placed in plastic trays covered with PVC film for 7 days to maintain high humidity; after 30 days of cultivation, they were transferred to larger trays and kept in a greenhouse.

2.3. Free polyamine analysis

The analysis of free polyamines was carried out using high-performance liquid chromatography (HPLC - Shimadzu, Japan) as